



Prevalence of feline immunodeficiency virus and feline leukaemia virus in domestic cats in Hungary

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Abstract

Objectives Feline immunodeficiency virus (FIV) and feline leukaemia virus (FeLV) are retroviruses affecting cats worldwide. The objectives of the study were to estimate the prevalence of these retroviruses in domestic cats in Hungary and to characterise the phylogenetic relationships of FIV strains.

Methods A total of 335 anticoagulated whole-blood samples obtained from both a healthy and ill cat population were examined for the presence of FIV and FeLV with two methods: ELISA and PCR. Statistical analysis was carried out to analyse the data obtained. Sequencing and phylogenetic analysis of partial polymerase (*pol*) gene sequences was performed to describe circulating FIV subtypes.

Results Statistical analysis showed 11.8% and 9.9% true prevalence of FeLV and FIV, respectively, with ELISA. The apparent prevalence calculated from the PCR results were 17.3% for FeLV and 13.1% for FIV. Phylogenetic analysis of partial *pol* gene sequences obtained from 22 FIV strains showed that all observed Hungarian strains belonged to FIV subtype B. The strains were grouped into several monophyletic subgroups reflecting the geographic locations of the origin of the samples. The overall mean genetic similarity between the analysed strains was 98.2%.

Conclusions and relevance We report the first thorough overview of the prevalence of FeLV and FIV in Hungary, which is relatively high, and give insight into the genetic diversity of Hungarian strains of FIV.

Keywords: Immunodeficiency virus; leukaemia virus; prevalence; phylogeny; Hungary

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Introduction

Feline leukaemia virus (FeLV) is an enveloped single-stranded RNA virus belonging to the *Retroviridae* family, genus *Gammaretrovirus*, and it infects feline species. The predominant subgroup, designated FeLV-A, represents the transmissible form of the virus spread cat to cat in nature and from which the other subgroups, FeLV-B,^{1,2} FeLV-C and FeLV-T,³ arise de novo during the course of infection.^{4–6} The infection is common worldwide and is transmitted by secretions via the oronasal route, predominantly by prolonged contact, but also by direct inoculation.⁷

Feline immunodeficiency virus (FIV) is a member of the *Lentivirus* genus within the *Retroviridae* family and it

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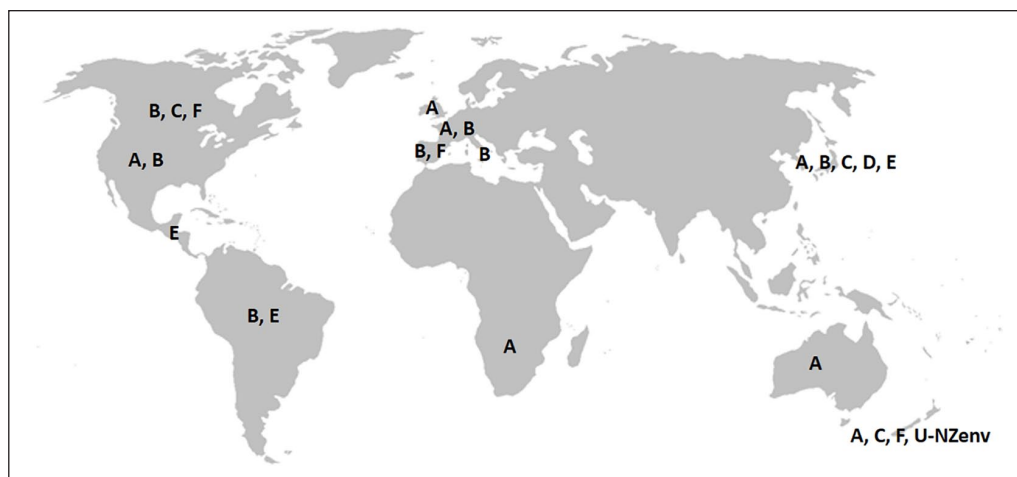


Figure 1 Worldwide distribution of feline immunodeficiency virus subtypes (map scheme: www.outline-world-map.com). Subtypes A and B are spread widely, subtypes C, D, E, F and U-NZenv are distributed only regionally

infects species of the families Felidae and Hyaenidae.⁸ It causes an acquired immune deficiency syndrome (AIDS) in cats, resembling AIDS caused by HIV in humans. Transmission is usually by direct inoculation (eg, bite and scratch wounds). The strains are grouped into seven phylogenetic subtypes A–F and U-NZenv.^{9–19} Distribution of discovered subtypes is illustrated in Figure 1.^{11,19–25}

To test the infection status of cats, point-of-care ELISA tests are widely used, which detect antibodies against the p24 protein of FIV and the p27 antigen of FeLV. The most frequent test used to confirm ELISA results, or in case of false/non-interpretable results, is PCR.²⁶

Studies report a relative low prevalence of these viruses worldwide. In North America, FeLV prevalence ranges between 2.3% and 7.5%, and is 2% in Australia, whereas in Europe it is slightly higher (3.6–15.6%). FIV prevalence levels are quite similar: 2.5–7.5% in North America, 15% in Australia and 3.2–8.3% in Europe.^{27–37}

The aim of this study was to estimate the prevalence of these retrovirus infections in domestic cats in Hungary, to evaluate the main factors affecting the infection rate and to examine the phylogenetic relations of the FIV strains detected.

Materials and methods

A total of 335 randomly selected, client-owned domestic cats, presented in 24 clinics, over a period of 3 years (2016–2018) were tested from all over Hungary (Figure 2). No free-roaming or sheltered cats were included in the survey. Age, sex, patient history, vaccination status and the household status of cats were registered. General physical examination was performed in each case and EDTA-anticoagulated blood samples were drawn as a part of the routine diagnostic or screening process. A Witness FeLV-FIV ELISA test (Zoetis) was performed

immediately, according to the manufacturer's instructions. The rest of the blood samples were frozen and sent to the Department of Pathology, at the University of Veterinary Medicine, Budapest, and they were stored at -80°C until further examination.

Serology testing

The point-of-care tests used in this study detect the presence of FeLV p27 antigen (92.9% sensitivity and 96.5% specificity) and FIV antibodies against p24 antigen (93.8% sensitivity and 93.4% specificity).^{38,39} Sensitivity and specificity values were given according to data provided by Zoetis (used in the statistical analysis), although slightly different values can be found in some field studies (eg, 89.0%/95.5% for FeLV and 94.7%/100% for FIV).⁴⁰ One drop (0.05 ml) of EDTA-anticoagulated whole blood was used as per the manufacturer's instructions (Zoetis).

PCR

From the stored whole-blood specimens, nucleic acid extraction was carried out in a QIAcube instrument (Qiagen) with the use of QIAmp cadior Pathogen Mini Kit (Qiagen), according to the manufacturer's instructions. Nucleic acid was eluted in 60 μl RNase-free distilled water (Qiagen). The preparation of endpoint PCR for FIV, TopTaq Master Mix Kit (Qiagen) was used in accordance with manufacturer's instructions: 25 μl master mix, 0.5 μl forward primer (40 μM), 0.5 μl reverse primer (40 μM), 5 μl CoralLoad Concentrate, 18 μl RNase-free water and 1 μl template DNA, and were mixed together for each sample. The hot-start PCR amplification with the given protocol was carried out as follows: 95 $^{\circ}\text{C}$ for 15 mins; 95 $^{\circ}\text{C}$ for 45 s, 60 $^{\circ}\text{C}$ for 45 s and 72 $^{\circ}\text{C}$ for 1 min (40 cycles); and 72 $^{\circ}\text{C}$ for 15 mins. The FIV primers used in the study amplify early reverse transcription products (process of reverse transcription

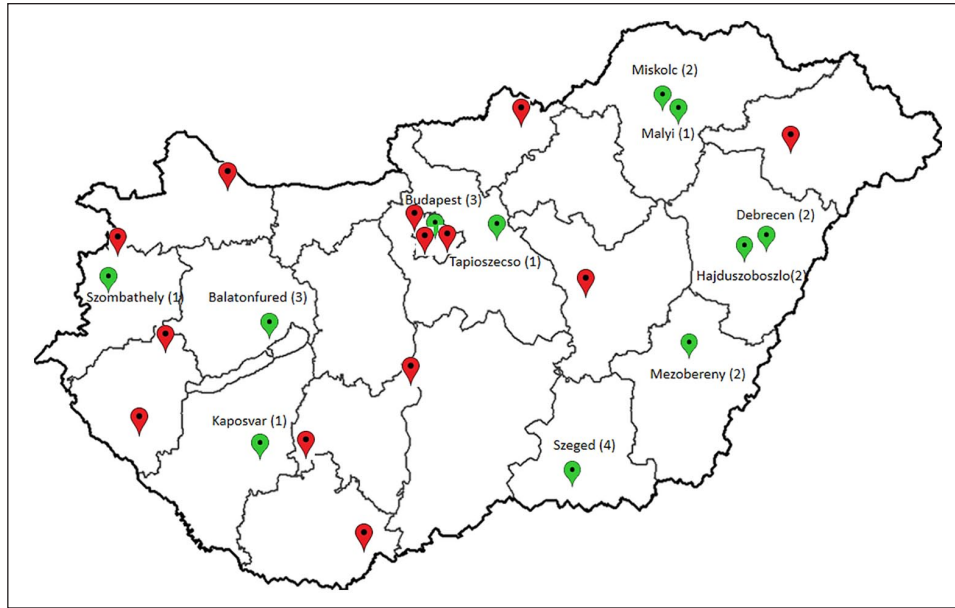


Figure 2 Origin of the samples collected in this study (map scheme: www.terkepek.net). On the map of Hungary, the 24 locations from where the blood samples were taken are indicated (red and green pinpoints). Cities highlighted with green pinpoints are the locations of partially sequenced FIV strains. Beside the name of city, number in brackets indicate how many sequences were obtained from that location

RNA to DNA); both bind in the long terminal repeat (LTR) region, which enables the detection of low-dose and/or recent infections (primer sensitivity was 10^2 – 10^3 cell-associated virus).^{41,42} For the detection of FeLV, a One Step RT-PCR Kit (Qiagen) was used. A master mix containing 5.7 μ l RNase-free water, 2 μ l 5 \times buffer, 0.4 μ l dNTP, 0.4 μ l enzyme mix, 0.1 μ l RNase inhibitor, 0.2 μ l forward primer (40 μ M) and 0.2 μ l reverse primer (40 μ M) were added into tubes for each sample (0.5 μ l each). The amplification was 50°C for 30 mins and 94°C for 3 mins (reverse transcription and initial denaturation); 95°C for 15 s, 60°C for 1 min and 72°C for 1 min (45 cycles); and 72°C for 10 mins. FeLV primers were obtained from a publication.⁴³ The 163 base pair (bp) (FIV) and 150 bp (FeLV) long amplicons were visualised by electrophoresis in 1.5% agarose gel.

Statistical analysis

Statistical calculations were performed in an R environment (R Core Team, Vienna, Austria) and a logistical regression model was used to detect the relationship between the examined variants and retroviral infection status.⁴⁴ The Epi.prev function was used to calculate prevalences.⁴⁵ Cohen's kappa (κ) was counted to show possible cross-compliance between the ELISA and PCR methods.⁴⁶

Phylogenetic analysis

In FIV-positive cases, partial proviral *pol* genes were amplified with the previously described protocol of Adams et al.⁴⁷ PCR products were subjected to gel

electrophoresis, amplicons of 576 bp length were cut out manually and purified by a Qiagen Gel Extraction Kit (Qiagen). Bidirectional Sanger sequencing reaction was performed with the corresponding primers and the capillary electrophoresis was made by a commercial provider (Microsynth). In two cases (I/14/16 and II/7/8) repeated sequencing did not result in clean sequences; thus, these PCR products were cloned into a pJET1.2 blunt vector and subsequently transformed into *Escherichia coli*, with the help of a CloneJET PCR Cloning Kit (ThermoFisher Scientific).

After proofreading of the obtained sequences, they were assembled using the E-INS-i method of the online software MAFFT version 7,⁴⁸ and aligned against available FIV genomes downloaded from GenBank that represent the overall diversity of the virus. Maximum likelihood analyses were carried out and the trees were visualised and edited with MEGA7⁴⁹ using the Tamura-Nei model with 1000 bootstrap replicates and gamma distribution.⁵⁰ Pairwise genetic analyses were conducted using the Kimura 2-parameter model in MEGA7.⁵¹

Results

Altogether, samples from 335 domestic cats were included in the study. All cats had owners – no stray or shelter animals were included. The mean age of cats in this study was 4.9 years (range 5 months to 18 years). A total of 155 females (46.3%) and 180 males (53.7%) were included in the research. Of the 155 females, 90 (58.1%) were intact and 65 (41.9%) were spayed; of the 180 males, 119 (66.1%)

were intact and 61 (33.9%) were castrated. Seventy-seven cats were kept strictly indoors (23.0%) and 258 had outdoor access (77.0%). A total of 136 cats were found to be clinically healthy during the physical examination. We observed an overall low prevalence of vaccination: only 98 (29.3%) were immunised at some point of their lifespan, usually with a combined vaccine, and among these cats, only 39 (11.6%) were vaccinated against FeLV (however, two of them proved to be FeLV-infected).

Of the 335 samples, 44 (13.1%) and 51 (15.2%) were positive for FeLV and FIV, respectively, with the Witness ELISA test kit. Using subsequent PCR tests, 58 (17.3%) were positive for FeLV and 47 (14.0%) for FIV. FeLV–FIV coinfection was observed in 10 (0.03%) cats. In some cases, only the ELISA test or the PCR test was positive. In the case of FIV, 11/51 ELISA tests and 4/47 PCR test results were positive, when the parallel assay was negative. In the case of FeLV, 4/44 ELISA results and 14/58 PCR results were single-positive.

Total apparent prevalence, counted from data obtained with the Witness test, was 14.03 (95% confidence interval [CI] 10.72–18.16) in the case of FeLV and 15.22 (95% CI 11.77–19.46) in the case of FIV. True prevalence was also calculated with the previously estimated sensitivity and specificity of the Witness test: 11.78 (95% CI 8.08–16.4) for FeLV and 9.89 (95% CI 5.93–14.75) for FIV.

Apparent prevalences calculated from the PCR results were 17.31 (95% CI 13.64–21.73) and 13.13 (95% CI 9.93–17.17) for FeLV and FIV, respectively. Cross-compliance was analysed by calculating Cohen's κ coefficient. This coefficient was 0.786 (95% CI 0.68–0.87) for FeLV ($P < 0.05$) and 0.816 (95% CI 0.72–0.9) for FIV ($P < 0.05$). Both results indicate a significant coherence between the Witness ELISA and PCR results.

With logistic regression, a 2.85 (95% CI 1.19–8.49; $P = 0.033$) times higher chance of infection with FeLV and a 4.14 (95% CI 1.62–14.07; $P = 0.0083$) times higher chance of FIV was found in correlation with outdoor access, according to the Witness test results. Calculating the PCR results, a 2.51 (95% CI 1.156–6.29; $P = 0.0306$) and a 3.41 (95% CI 1.32–11.64; $P = 0.0235$) times higher possibility was found in correlation with FeLV and FIV infection, respectively.

No significant correlation was found with the animals' age regarding FeLV infection (either with results of ELISA or PCR), but a 1.12 times higher chance (both with the Witness test and PCR [95% CI 1.04–1.19 ($P = 0.0012$) for ELISA] and [95% CI 1.04–1.19 ($P = 0.0014$) for PCR]) was observed in the case of FIV infection for every year older the cats were. We could not find significant correlation of infection status with sex (although males were 1.32 times more likely to have FIV and/or be FeLV-infected than females [95% CI 0.71–2.5, $P = 0.3872$]). The same was observed for neutering status: there was a 1.34 times higher risk for intact males to be infected (95% CI 0.57–3.42; $P = 0.5187$) and a 0.78 higher risk for intact

females (95% CI 0.3–2.08; $P = 0.609$), but these data were not statistically significant.

Altogether, we were able to sequence 22 partial *pol* genes out of the 47 FIV PCR-positive cases. We suspect that the rest of the samples contained insufficient amounts or poor quality nucleic acids. Multiple alignments and maximum likelihood tree reconstructions revealed that all of the strains were clustered into subtype B (Figure 3). Within clade B, the Hungarian sequences were located into a monophyletic group that also included a strain originating from Joinville, Brazil, 2017 (acc. number: KY629414.1). Within this 'Hungarian' cluster, the strains formed several subgroups of closely related sequences that mostly reflected the geographic location of their origin. The subgroups were supported by relatively high bootstrap values.

Pairwise genetic analyses revealed that the overall mean genetic similarity between the analysed strains was 98.2% (the lowest similarity was 88.1% and the highest was 99.8%). Only two strains originated from indoor cats and the majority of these cats were males (16 males, six females). The samples represent most of geographic regions of Hungary (Figure 2). GenBank accession numbers are MN401425–MN401446.

Discussion

Our results show a relatively high rate of infection of FeLV in owned domestic cats in Hungary vs the most recent data from surrounding (or more distant) countries (with the exclusion of prevalence data including free-roaming or shelter cats). The prevalence rate of FIV does not show such a difference.²⁰ Data regarding vaccination status showed that there is a strong need for improving in the vaccination prevalence in Hungarian cat populations. Two of the immunised cats had FeLV-positive results, both determined by ELISA and PCR, but their vaccinations were not regular, which could be an answer to possible unprotected periods of time and the ability to become infected.⁵² Moreover, 77.0% of cats had outdoor access and most of them were not neutered. Previous publications and our statistics show a higher occurrence of retroviral infection in intact (usually male) outdoor cats.⁵³

Another possible explanation for the higher observed prevalence data could be the weak positive predictive value of every diagnostic test that does not have 100% specificity in the case of low prevalence rates in a population ('false positive paradox').

In relation to the discordant results between ELISA and PCR in some cases, where only one test method was positive, there can be various explanations. In the case of FIV, the Witness test detects antibodies, whereas the PCR amplifies and detects part of the LTR region of the virus. In the early stages of infection there are no detectable antibodies (most cats produce antibodies within 8 weeks of exposure);⁵⁴ moreover, there might be extremely low

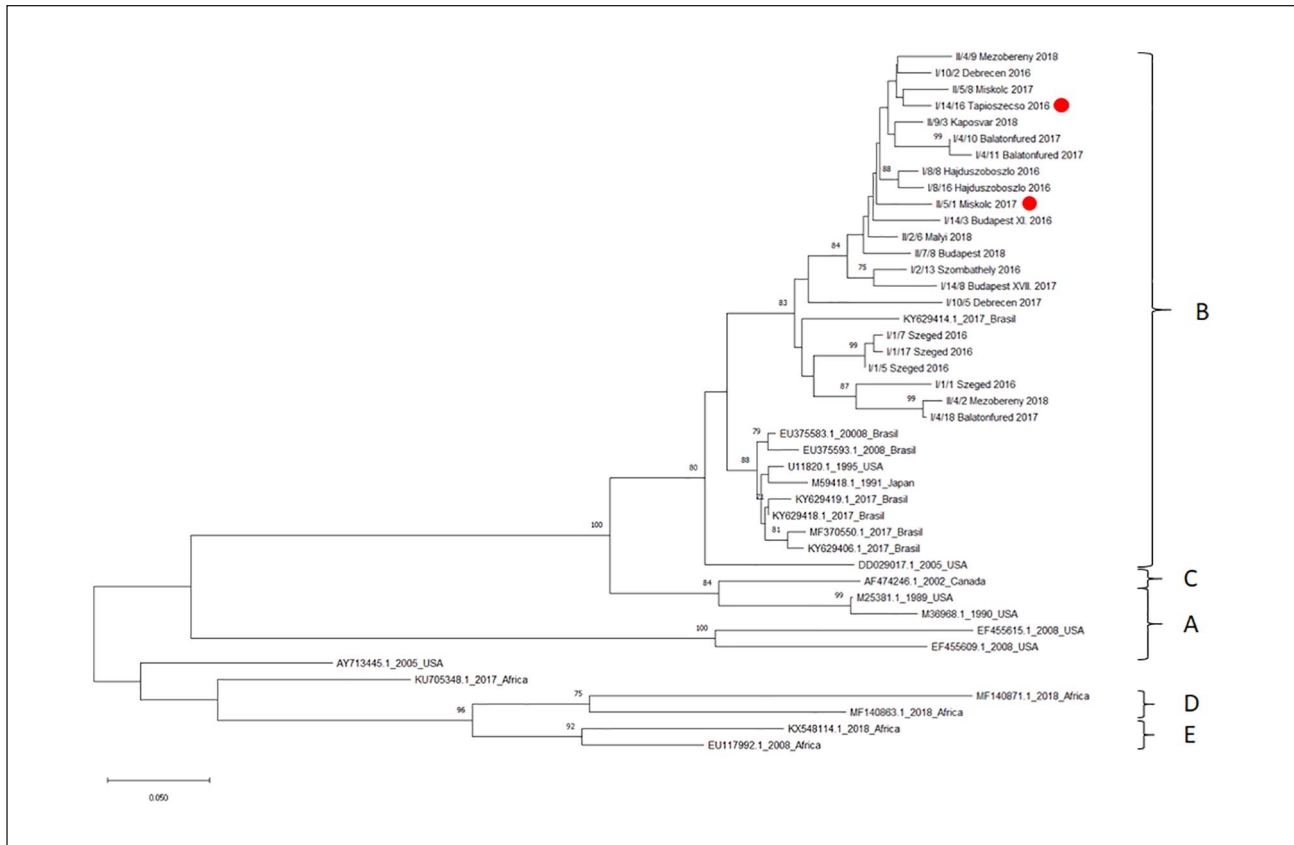


Figure 3 Phylogenetic tree reconstructed from the partial *pol* gene sequences of Hungarian feline immunodeficiency virus strains and selected reference sequences from GenBank. Maximum likelihood bootstrap support values (≥ 70) are shown as percentages above the branches. The two indoor cats are marked with red dots; all the others had outdoor access. Strains displayed on the phylogenetic tree are coded differently in the case of Hungarian strains, the code of sample and city of the cat's origin are shown. In case of reference sequences obtained from GenBank, accession number, year of publication and country of collection are displayed

levels of antibodies in the terminal phase of the disease, causing a false-negative ELISA result, whereas the PCR result will be positive as a result of increased viral load in the circulation.³⁷ However, a positive ELISA and negative PCR result can suggest extremely low levels of circulating proviral DNA/viral RNA (which can be the case in the asymptomatic phase of FIV infection) or viral genetic diversity (where nucleotide sequence dissimilarity can result in failure of primer binding).³⁷ The presence and interference of maternal antibodies with the ELISA can be excluded in our cases, as the youngest animals included in the study (5–6 months of age) were FIV negative. Maternal antibodies can be present in kittens up to 6 months of age.⁵⁵ In the case of FeLV, the Witness test detects the p27 antigen, whereas the PCR amplifies and detects the U3 part of the LTR region. A negative ELISA and positive PCR result can also suggest an early phase of infection or illness in a regressive stage, when small amounts of proviral DNA/viral RNA are already present in the circulation, but the amount of the antigen is still below detectable levels.

Regarding the phylogenetic analysis of the FIV sequences, all the sequenced samples belonged to

subtype B, and they form a unique cluster of strains that includes Hungarian sequences and also a Brazilian one. The monophyletic pattern suggests a common ancestor for all the Hungarian (and the single Brazilian) sequences analysed in the study. Based on our data, it can be speculated that there was a single event of virus introduction into the country and the genetic diversity observed is the result of divergent local evolution of the strains.

It must be highlighted that our 22 sequences are insufficient to give a complete picture of the circulating FIV strains in Hungary, but it can be suggested that mainly subtype B strains are present in Hungary. This result is largely in harmony with data published from surrounding countries, where mostly subtypes A and B were discovered.²⁰

Conclusions

This is the first report of a thorough investigation on retroviral prevalence and phylogenetic analyses of FIV strains in Hungary, and suggests that FIV subtype B is the most prevalent in this country. This data set and prospective phylogenetic analyses of further FIV

strains can give useful information for vaccine developers as the only existing FIV vaccine, which is not available in Hungary (Fel-O-Vax FIV; Boehringer Ingelheim), contains inactivated whole virus subtypes A and D, which will not confer a sufficient protective effect against subtype B.^{21,56}

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Conflict of interest The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval This work involved the use of non-experimental animals only (owned or unowned), and followed internationally recognised high standards ('best practice') of individual veterinary clinical patient care. Ethical approval from a committee was not necessarily required.

Informed consent Informed consent (either verbal or written) was obtained from the owner or legal custodian of all animal(s) described in this work for the procedure(s) undertaken. No animals or humans are identifiable within this publication, and therefore additional informed consent for publication was not required.

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